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The Potato MAP3K StVIK Is Required for the *Phytophthora infestans* RXLR Effector Pi17316 to Promote Disease[1[OPEN]]

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Plant pathogens deliver effectors to manipulate processes in their hosts, creating a suitable environment for invasion and proliferation. Yet, little is known about the host proteins that are targeted by effectors from filamentous pathogens. Here, we show that stable transgenic expression in potato (*Solanum tuberosum*) and transient expression in *Nicotiana benthamiana* of the arginine-any amino acid-leucine-arginine effector Pi17316 enhances leaf colonization by the late blight pathogen *Phytophthora infestans*. Expression of Pi17316 also attenuates cell death triggered by the pathogen-associated molecular pattern Infestin1 (INF1), indicating that the effector suppresses pattern-triggered immunity. However, this effector does not attenuate cell death triggered by a range of resistance proteins, showing that it specifically suppresses INF1-triggered cell death (ICD). In yeast two-hybrid assays, Pi17316 interacts directly with the potato ortholog of VASCULAR HIGHWAY1-interacting kinase (StVIK), encoding a predicted MEK kinase (MAP3K). Interaction in planta was confirmed by coimmunoprecipitation and occurs at the plant plasma membrane. Virus-induced gene silencing of *VIK* in *N. benthamiana* attenuated *P. infestans* colonization, whereas transient overexpression of *StVIK* enhanced colonization, indicating that this host protein acts as a susceptibility factor. Moreover, *VIK* overexpression specifically attenuated ICD, indicating that it is a negative regulator of immunity. The abilities of Pi17316 to enhance *P. infestans* colonization or suppress ICD were compromised significantly in *NbVIK*-silenced plants, demonstrating that the effector activity of Pi17316 is mediated by this MAP3K. Thus, StVIK is exploited by *P. infestans* as a susceptibility factor to promote late blight disease.

Plants have evolved a complex network of cross-talking pathways to sense and respond appropriately to their environment. These signaling networks are tightly regulated to allow plants to fine-tune responses and to limit energy expenditure to where it is needed most at any given time (Koornneef and Pieterse, 2008; Rojas et al., 2014). During plant immune responses, plants use plasma membrane (PM)-localized pattern recognition receptors (PRRs), including receptor-like kinases (RLKs), to detect the earliest signatures of foreign molecules or of cellular damage in the extracellular environment (Ingle et al., 2006; Zipfel, 2008). These receptors recognize a wide variety of microbe- and pathogen-associated molecular patterns (PAMPs) from different possible invading organisms in a family- or even species-specific manner. For example, transglutaminase GP42 (Nürnberger et al., 1994; Brunner et al., 2002), CELLULOSE-BINDING ELICITOR LECTIN (Mateos et al., 1997; Gaulin et al., 2006), and the elicitin Infestin1 (INF1; Derevnina et al., 2016) all can be detected from oomycetes; β-glucan (Klarzynski et al., 2000), chitin (Kaku et al., 2006), and ergosterol (Laquitaine et al., 2006) from fungi; and lipopolysaccharide, the translation elongation factor EF-Tu, and flagellin from gram-negative bacteria (Nürnberger et al., 2004; Nürnberger and Lipka, 2005). Recognition of these conserved microbial molecules activates pattern-triggered immunity (PTI).

The activation of PRRs usually requires a coreceptor for downstream signaling to trigger PTI via MAPK cascades (Ingle et al., 2006; Pitzschke et al., 2009). PRR coreceptors, such as the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE RLK family member BRASSINOSTEROID INSENSITIVE1 (BRI1)-Associated Receptor Kinase (BAK1), may play a role in activating multiple signal transduction pathways and could be a point of cross talk between different pathways. For example, BAK1 is involved in the perception of PAMPs to trigger PTI and the phytohormone brassinosteroid (BR) by BRI1 (He et al., 2000; Kemmerling et al., 2007; Chinchilla et al., 2009; Belkhadir et al., 2012). Typically, MAPKs are organized

in a hierarchical cascade in which a MAPK is activated by a MAPK kinase (MAPKK, MEK, or MAP2K), which itself is activated by a MAPKK kinase (MAPKKK, MEKK, or MAP3K; Rodriguez et al., 2010). MAPKs phosphorylate a number of different substrates, ranging from transcription factors to RESPIRATORY BURST OXIDASE HOMOLOG D, which produces reactive oxygen species to induce defense responses (Asai et al., 2008). The Arabidopsis (*Arabidopsis thaliana*) genome encodes 20 MAPKs, 10 MAP2Ks, and more than 80 MAP3Ks (Pitzschke et al., 2009). MAP3Ks in plants are a heterogenous group that can be divided into three additional subgroups. These are the MEKK (MAPK/ERK kinase kinase)-like subgroup, which are mainly organized in linear cascades and for which functional evidence exists that they act as MAP3Ks in planta; and the Raf-like and ZIK-like subgroups, which have a wide range of protein substrates and for which functional characterization comes largely from organisms other than plants (MAPK Group, 2002; Colcombet and Hirt, 2008). MAPK activation in PTI results in, among other things, the phosphorylation of specific downstream transcriptional activators (e.g. WRKY transcription factors) that induce the expression of defense genes, such as those producing antimicrobial peptides (Ingle et al., 2006; Pitzschke et al., 2009).

One of the best characterized PTI signaling pathways follows the recognition of flg22 (a 22-amino acid epitope from bacterial flagellin). In Arabidopsis, the RLK FLAGELLIN SENSITIVE2 (FLS2) complexes with BAK1 to activate MAPK signaling (Chinchilla et al., 2006, 2007). Early investigations found that the MAP3K MEKK1 activates the MAP2Ks MKK4 and MKK5, which, in turn, activate the MAPKs MPK3 and MPK6 to positively regulate PTI responses. Genetic studies implicate a second cascade involved in flg22 perception, consisting of MEKK1-MKK1/2-MPK4, that negatively regulates plant defenses responses, although MEKK1 has not been shown to interact with FLS2 directly (Suarez-Rodriguez et al., 2007; Gao et al., 2008). The cross talk between the positive regulators MPK3/6 and the negative regulation by MPK4 indicates that the plant tightly controls defense responses to respond appropriately to environmental challenges (Suarez-Rodriguez et al., 2007).

Recognition of INF1 has been shown to be via a receptor-like protein known as Elicitin-Response Receptor (ELR) in potato (*Solanum tuberosum*), and since ELR has no kinase domain itself, it is hypothesized to form complexes with other kinase domain-containing proteins, such as SUPPRESSOR OF BIR1-1 and BAK1, to transduce the signal (Du et al., 2015). INF1-triggered cell death (ICD) is BAK1 dependent (Chaparro-Garcia et al., 2011). As with flg22, the detection of INF1 activates two MAPK cascades. One cascade involves a currently unknown MAP3K and then the nucleus-localized *Nicotiana benthamiana* MAP2K MKK1/MEK2 (Takahashi et al., 2007; Asai et al., 2008), which interacts physically with salicylic acid-induced protein kinase (Zhang and Klessig, 1997). Interestingly, *N. benthamiana* MEK2 also is upstream of two other *N. benthamiana* MAPKs (Yang et al., 2001), wound-induced protein kinase (Seo et al., 1995, 1999) and NTF4 (Ren et al., 2006). A second cascade triggered by INF1 also has been identified that activates the MAP3K NPK1 (Jin et al., 2002; Soyano et al., 2003) and, subsequently, the MAP2K MEK1 and the MAPK NTF6 (Asai et al., 2008).

It is commonly believed that well-adapted plant pathogens and symbionts have evolved methods to evade or overcome the PTI response in host plants in order to successfully establish infection (Schulze-Lefert and Panstruga, 2011). A key strategy is to deploy small, secreted proteins, known as effectors, into the apoplastic space between cells or to deliver them inside plant cells (Toruño et al., 2016). Therefore, effectors play a critical role in aiding pathogenic organisms to overcome PTI, leading to effector-triggered susceptibility. Every species of plant-interacting microbe has evolved at least one system to secrete effector proteins and possesses a unique combination of effector proteins for the suppression of immunity in a particular host or hosts. There are many examples of bacterial effectors that suppress PTI, such as the BAK1-targeting effectors AvrPtoB and HopF2 (Lu et al., 2010; Zhou et al., 2014) from *Pseudomonas syringae* pv *tomato* DC3000 and the phosphothreonine lyase HopA1, which targets the key Arabidopsis MAPKs MPK3, MPK4, and MPK6 (Zhang et al., 2007). Oomycete effectors that suppress PTI responses also have been identified. The *Phytophthora infestans* cytoplasmic Arg-any amino acid-Leu-Arg (RXLR) effector Pi18215/SUPPRESSOR OF EARLY FLG22-INDUCED IMMUNITY7 (SFI7), in addition to suppressing flg22-mediated MAPK activation, also can partially suppress ICD (Zheng et al., 2014). ICD also can be suppressed in *N. benthamiana* by additional *P. infestans* effectors: AVR3a, through modulation of the

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P.R.J.B. and E.M.G. conceived the research and designed the experiments; P.C.B. supervised cell biology experiments; Z.T. supervised transgenic potato experiments; W.Z. performed potato transformations; M.A. performed the original yeast two-hybrid screen; L.M.G. performed early cloning and functional analyses; F.M. and Q.H. performed most of the experiments; E.M.G., F.M., and Q.H. performed data analysis and made figures; E.M.G., P.R.J.B., and F.M. wrote the article with contributions of all the authors.

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ubiquitin E3 ligase CMPG1 (Bos et al., 2010; Gilroy et al., 2011); Pi02860, through manipulation of NRL1, another E3 ligase (Yang et al., 2016); and PiAVR2, through activating the BR pathway (Turnbull et al., 2017). AVR3a also can suppress cell death triggered by the detection of the *Cladosporium fulvum* effectors AVR4 and AVR9 by tomato (*Solanum lycopersicum*) receptors Cf4 and Cf9, respectively (Gilroy et al., 2011). Intriguingly, PexRD2, a *P. infestans* RXLR effector that targets MAP3Kε, can suppress the BAK1-dependent Cf4/AVR4 cell death but not ICD (King et al., 2014; Postma et al., 2016).

Recently, some *P. infestans* RXLR effectors were shown to target negative regulators of immunity that could be described as susceptibility (S) factors (Boevink et al., 2016a; Whisson et al., 2016). These include Pi04089, which targets the RNA-binding protein K-homology RNA-binding protein1 (Wang et al., 2015); Pi04314, which targets three host protein phosphatase catalytic isoforms (Boevink et al., 2016b); and Pi02860, which interacts with the predicted CULLIN3-associated ubiquitin E3 ligase NRL1 in order to suppress ICD and promote infection (Yang et al., 2016). More recently, we also showed that PiAVR2 up-regulates a BR-responsive basic helix-loop-helix transcription factor that suppresses immunity and is required for infection (Turnbull et al., 2017).

In this study, we show that transient expression of the RXLR effector PITG_17316 (Pi17316) in the model host *N. benthamiana*, or stable transformation in potato cv E3, enhances leaf colonization by *P. infestans*. Expression of the effector in *N. benthamiana* revealed that it localizes to the PM and suppresses ICD. Pi17316 interacts with a potato MAP3K that is a candidate ortholog of Arabidopsis VASCULAR HIGHWAY1 (VH1)-INTERACTING KINASE (VIK) in a yeast two-hybrid (Y2H) library screen and in planta by coimmunoprecipitation and bimolecular fluorescence complementation (BiFC) assays. Virus-induced gene silencing (VIGS) of *NbVIK* attenuated *P. infestans* colonization of *N. benthamiana*. In contrast, overexpression of potato StVIK enhances *P. infestans* colonization and suppresses ICD, indicating that StVIK is a negative regulator of plant immunity. Importantly, VIGS of *NbVIK* prevented the suppression of ICD by Pi17316, whereas ICD suppression by AVR3a was unaltered. Finally, Pi17316 could no longer enhance *P. infestans* colonization in the *NbVIK*-silenced background, indicating that VIK is required for the effector to assist infection and, thus, that it is an S factor exploited by the pathogen.

RESULTS AND DISCUSSION

Pi17316 Promotes *P. infestans* Virulence in *N. benthamiana* and Potato

The gene *Pi17316* (*PITG_17316*) is annotated in the *P. infestans* genome as encoding a secreted RXLR-type effector protein (Haas et al., 2009). Consistent with other RXLR effectors, *Pi17316* is up-regulated by distinct genotypes of *P. infestans* during the biotrophic phase of infection on potato plants (Haas et al., 2009; Cooke et al., 2012; Supplemental Fig. S1). *Pi17316* was cloned (excluding the signal peptide) into a destination vector that fuses GFP to the N terminus and coexpressed with an empty vector (EV) expressing free monomeric Red Fluorescent Protein (mRFP). Intact GFP-Pi17316 (Supplemental Fig. S2) was observed to localize at the plant PM, whereas free mRFP was cytosolic (Fig. 1, A and B).

As demonstrated previously for other RXLR effectors (McLellan et al., 2013; Boevink et al., 2016b; Yang et al., 2016; Turnbull et al., 2017), transient expression of GFP-Pi17316 in *N. benthamiana* was found to promote significantly faster developing *P. infestans* lesions compared with an empty GFP control (ANOVA, $P < 0.001$; Fig. 1C). To explore this phenomenon further in potato plants, transgenic potato cv E3 lines were generated with constitutive 35S promoter-driven expression of Pi17316. Two cv E3 lines (A4 and A5) with detectable *Pi17316* transcript levels were selected (Supplemental Fig. S3A). These plants were challenged with a mixture of sporangia from two contemporary *P. infestans* isolates (HB0914-2 and HB0916-2) as described by He et al. (2015) to investigate the impact of constitutive *Pi17316* overexpression on colonization compared with cv E3 control plants at 5 dpi. *P. infestans* lesion size was enhanced significantly on both transgenic potato lines compared with the cv E3 control (Fig. 1D; Supplemental Fig. S3B).

Pi17316 Specifically Suppresses ICD

RXLR effectors can attenuate distinct defense signaling pathways in planta (Bos et al., 2010; Gilroy et al., 2011; King et al., 2014; Whisson et al., 2016). GFP-Pi17316 was tested to determine if it suppressed cell death signaling activated by two characterized pathways: ICD and the cell death triggered by coexpression of the *C. fulvum* effector AVR4 and its cognate tomato resistance protein Cf4. AVR3a was found to suppress both ICD and Cf4/AVR4 cell death along with at least one other cell death signaling event initiated at the plant PM (PTO/AvrPTO). However, AVR3a had no impact on ETI triggered by R3a/AVR3 or Rx/PVX Coat Protein (Gilroy et al., 2011). The expression of GFP-Pi17316 was found to significantly suppress ICD. However, unlike AVR3a, Pi17316 had no significant effect on Cf4/AVR4-triggered cell death (Fig. 2). This result is similar to that of another RXLR effector, Pi02860, which can suppress ICD but not Cf4/AVR4-triggered cell death (Yang et al., 2016). In addition, Pi17316 was unable to suppress cell death triggered by R3a/AVR3a, Rx/PVX-CP, or PTO/AvrPTO (Supplemental Fig. S4). To further test the potential suppression of PTI activated in the absence of cell death, we treated leaves of the control cv E3 and the 35S:Pi17316 transgenic potato lines A4 and A5 with flg22 and investigated the early-responsive

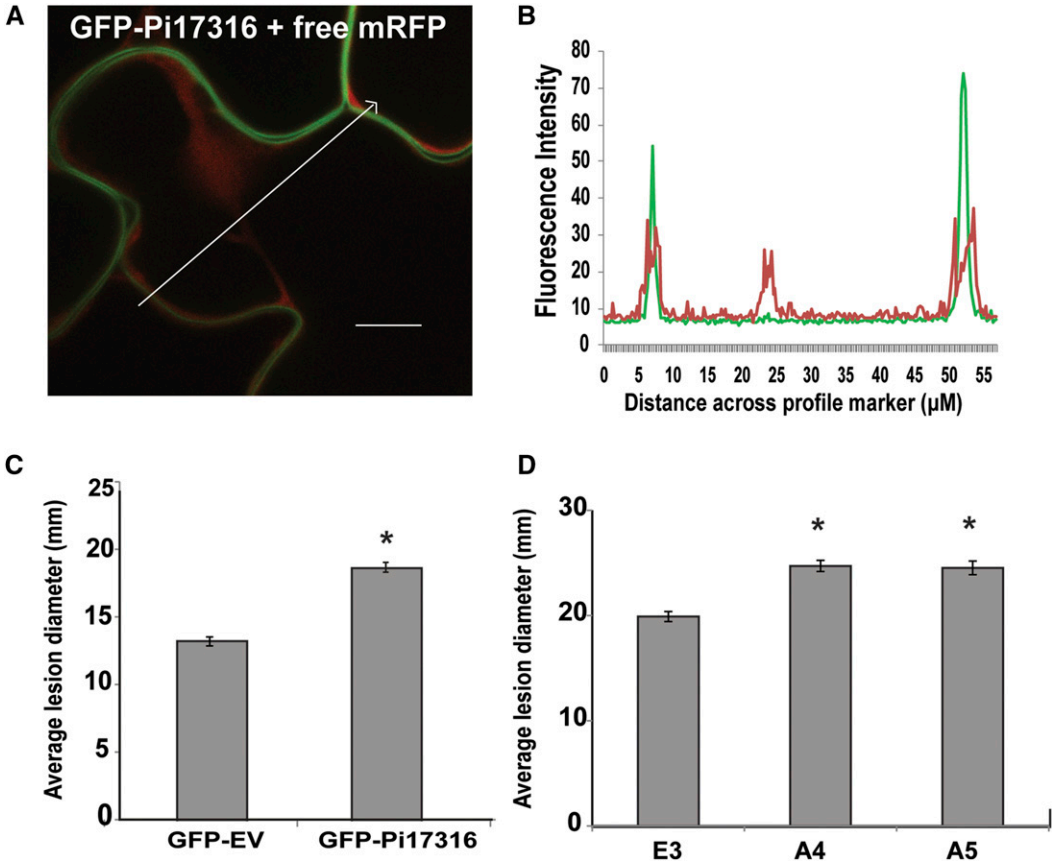

Figure 1. Localization of GFP-Pi17316 and enhancement of *P. infestans* colonization. A, Transient coexpression of GFP-Pi17316 with an mRFP-EV construct as a cytoplasmic marker. Bar = 20 μm. B, A plot of the profile indicated by the arrow in A shows GFP fluorescence (green line) and mRFP signal (red line). C, Transient expression of GFP-EV and GFP-Pi17316 followed by the addition of a concentrated suspension of *P. infestans* isolate 88069 sporangia measured at 7 to 8 d post inoculation (dpi; mean of five repetitions, 30 leaves per construct). *, Significant difference by one-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method ($P \leq 0.001$). D, Mean *P. infestans* lesion diameter of cv E3 and transgenic Pi17316-expressing potato plants (A4 and A5) measured at 5 dpi (three repetitions, 40 leaves per line). *, Significance difference by one-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method ($P \leq 0.001$). Error bars in C and D show SE.

genes *StWRKY7*, *StWRKY8*, and *StACRE31* as described previously (McLellan et al., 2013; Boevink et al., 2016b). Transcripts of the three genes accumulated to similarly high levels in cv E3, A4, and A5 only 30 min after flg22 treatment (Supplemental Fig. S5). It can be concluded that Pi17316 does not suppress early flg22-responsive gene induction, suggesting that the function of Pi17316 may be to specifically suppress signaling pathway(s) triggered by the perception of elicitins such as *P. infestans* INF1 and does not extend to other tested PTI pathways or events that trigger cell death by the activation of cell surface receptors or NB-LRR resistance proteins.

Pi17316 Specifically Targets the Potato MAP3K, StVIK

To further explore the mechanism of Pi17316 action in plants, a Y2H screen was performed to search for any possible host protein targets of Pi17316 using a library made from RNA extracted from *P. infestans*-infected potato leaf material as described previously (Bos et al., 2010; McLellan et al., 2013; Wang et al., 2015; Boevink et al., 2016b; Yang et al., 2016). Pi17316 was cloned into a bait construct containing a GAL4 DNA-binding domain and screened against the GAL4 activation domain (prey)-containing library to a depth of 2.6×10^6 cotransformants. Eighteen positive clones recovered from selection plates yielded sequences with high homology to an Arabidopsis MAP3K, known as VIK (At1g14000; Ceserani et al., 2009). The high conservation between Arabidopsis, potato, and *N. benthamiana* VIK amino acid sequences is highlighted in Supplemental Figure S6. To investigate the specificity of the interaction between Pi17316 and StVIK, a pairwise Y2H assay was performed with the full-length StVIK prey clones against the Pi17316 bait or vice versa using two other RXLR effectors, PexRD2 (Pi11383) and SFI5 (Pi13628), as controls. PexRD2 targets another MAP3K in the cytoplasm, StMAP3Kε (King et al., 2014). SFI5 shows a similar PM localization to Pi17316 and also enhances *P. infestans* leaf

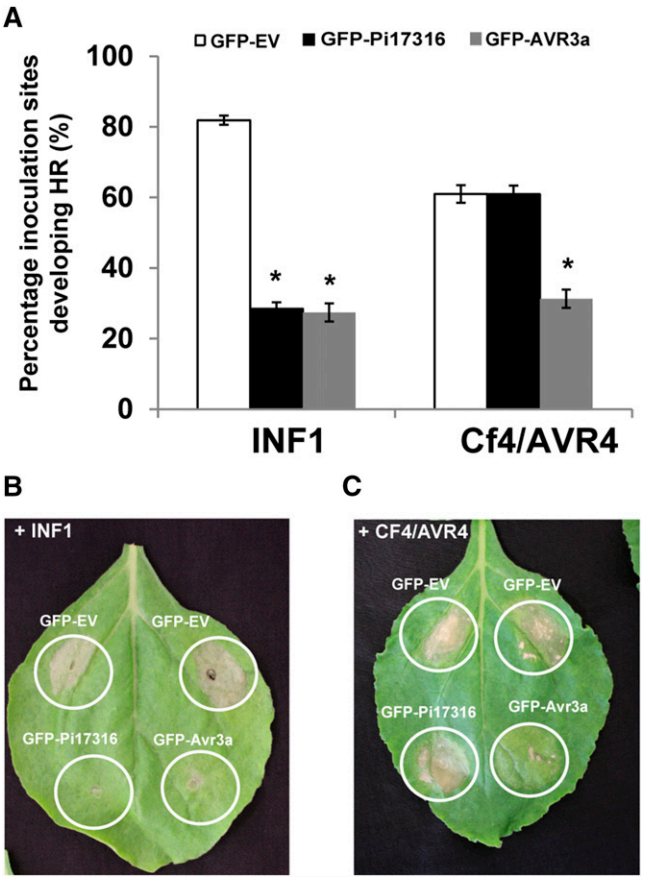

Figure 2. The effector Pi17316 inhibits ICD. A, Graph representing mean inoculation sites developing ICD and Cf4/AVR4 hypersensitive response (HR) lesions at 4 dpi when coinoculated with GFP-EV, GFP-Pi17316, and GFP-AVR3a as a control that suppresses both ICD and Cf4/AVR4. Data include five biological replicate experiments with six plants per replicate and four inoculations per plant; error bars show SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that both ICD + Pi17316 and ICD + AVR3a were significantly different from the GFP-EV (*, $P \leq 0.001$). Only GFP-AVR3a had a significant reduction in Cf4/AVR4 HR. B, Representative leaf image displaying ICD with GFP-EV, GFP-Pi17316, and GFP-AVR3a positive control at 5 dpi. C, Representative leaf image displaying Cf4/AVR4 HR with GFP-EV, GFP-Pi17316, and GFP-AVR3a positive control at 5 dpi.

colonization when transiently expressed in planta. Unlike Pi17316, SFI5 suppresses early transcriptional responses activated by the bacterial PAMP flg22 but does not suppress ICD (Zheng et al., 2014), suggesting that it does not share a similar function. While all yeast transformants grew on control plates, the interaction of Pi17316 with StVIK was indicated by the induction of β-galactosidase activity and growth on medium lacking His. The SFI5-VIK and PexRD2-StVIK combinations did not activate either reporter (Fig. 3). In addition, whereas PexRD2 interacted with StMAP3Kε, no such interaction was observed between Pi17316 and StMAP3Kε. These results indicate interaction specificity, in that Pi17316 does not generally target MAP3Ks and StVIK does not appear to be the target of another MAP3K-interacting effector, PexRD2.

StVIK is a Raf-like MAP3K and a member of subgroup C1 (MAPK Group, 2002). The C1 MAP3Ks are characterized by the presence of an N-terminal ankyrin repeat domain that facilitates protein-protein interactions with a diverse range of protein substrates, making a role in canonical MAPK cascades less likely (MAPK Group, 2002). MAP3K C1 family members also are mostly annotated as integrin-linked proteins with largely unknown function. However, one C1 family member, Integrin-Linked Kinase1 (ILK1), was shown recently to promote flg22 responses and resistance to bacterial pathogens in Arabidopsis (Brauer et al., 2016). Mammalian integrins have well-established roles in mediating the interaction between the extracellular matrix and the F-actin cytoskeleton (MAPK Group, 2002; Knepper et al., 2011), and the dysregulation of integrins is associated with many diseases and cancers (Sun et al., 2016; Paolillo and Schinelli, 2017). Arabidopsis NDR1 is an example of a plant integrin-like protein that plays well-characterized roles in ETI, in fluid loss, and in PM-cell wall adhesion in both PTI and broader stress responses (Knepper et al., 2011). Interestingly, GFP-StVIK transient expression was found to exhibit both cytoplasmic and nuclear localization (Supplemental Fig. S7, A and B), which could fit with the function of a protein with multiple and varied interaction partners.

For confirmation that specific interaction between Pi17316 and VIK occurs in planta, a coimmunoprecipitation experiment in *N. benthamiana* was performed by coexpressing GFP-StVIK with cMyc-Pi17316, cMyc-PexRD2, or cMyc-SFI5 using GFP-TRAP_M beads. GFP-StVIK and cMyc-labeled RXLR effector constructs were all stable when transiently expressed in planta, as indicated in the input samples. Only cMyc-Pi17316 was coimmunoprecipitated by GFP-StVIK, and not the cMyc-PexRD2 or cMyc-SFI5 controls (Fig. 4).

In order to determine where the interaction between Pi17316 and StVIK takes place within the plant cell, a BiFC assay was performed using PexRD2 and StMAP3Kε as controls. The C-terminal fragment of YFP (YC) was fused to StVIK while the N-terminal fragment (YN) was fused to Pi17316, to give YC-StVIK and YN-Pi17316, respectively. The fluorescence generated by BiFC between YN-Pi17316 and YC-StVIK occurred at the host cell PM (Fig. 5; Supplemental Fig. S7, C and D), whereas the fluorescence generated by the coexpression of YN-PexRD2 and YC-StMAP3Kε was cytoplasmic (Supplemental Fig. S7, E and F), as shown previously (King et al., 2014). In contrast, YFP fluorescence was barely detectable following the coexpression of either YN-PexRD2 with YC-StVIK or YN-Pi17316 with YC-StMAP3Kε (Fig. 5). The YN-Pi17316 and YC-StVIK fusion constructs were shown to be intact in planta by immunoblotting (Supplemental Fig. S7G). The failure to reconstitute YFP fluorescence by BiFC with Pi17316 and MAP3Kε confirms previous findings

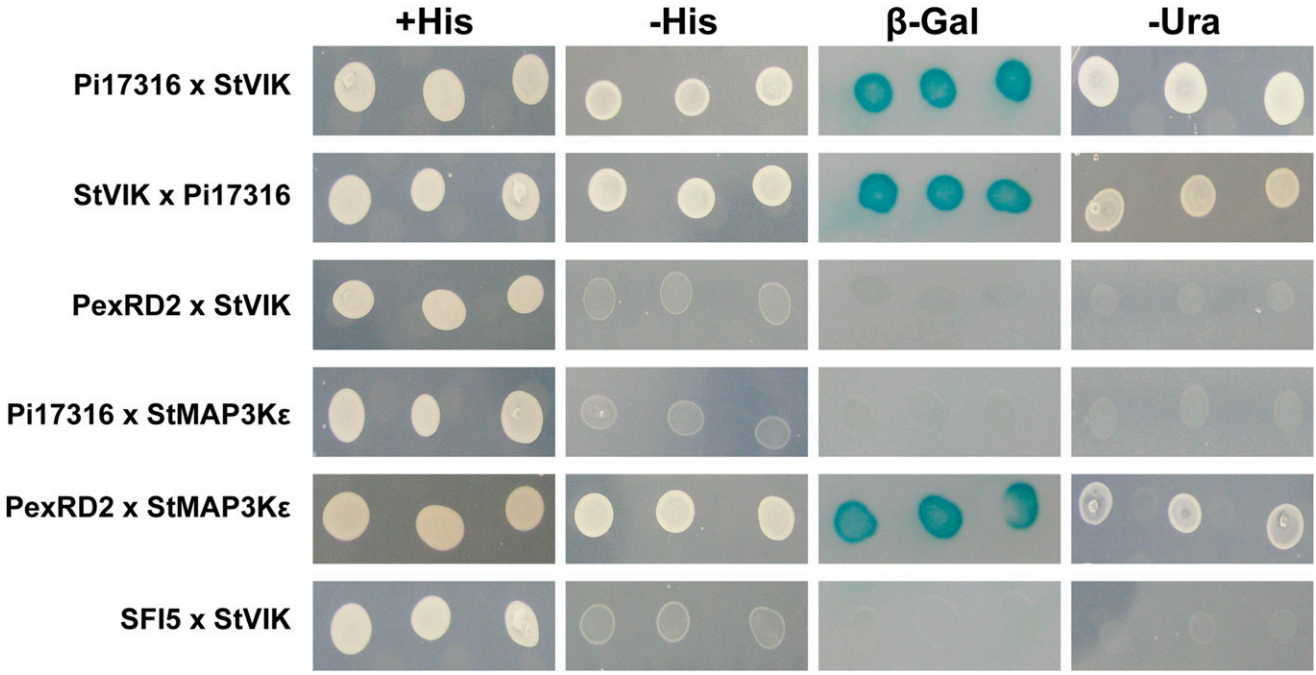

Figure 3. Pi17316 specifically targets the potato MAP3K, StVIK. Yeast coexpressing Pi17316 and the MAP3K StVIK, but not the target of PexRD2, MAP3Kε, grew on His selection (−His) medium, uracil selection (−Ura) medium, and yielded β-galactosidase (β-Gal) activity. In addition, another PM-localized effector, SFI5, does not interact with StVIK.

from the Y2H and coimmunoprecipitation experiments (Figs. 3 and 4). Figure 5D shows cells coexpressing GFP-Pi17316 and mRFP-StVIK to different levels. Whereas mRFP-StVIK shows cytoplasmic localization (cell 1) when coexpressed with a low level of GFP-Pi17316, a high level of GFP-Pi17316 (cell 2) results in a stronger association of mRFP-StVIK with the PM. The interaction of Pi17316 and StVIK at the PM connects to the putative function of VIK as an interactor of PM-associated RLKs, such as BRL2/VH1 (Ceserani et al., 2009), or other membrane-bound proteins, such as calmodulin-like proteins and transporters, as is the case for the related C1 family member ILK1 (Wingenter et al., 2011; Brauer et al., 2016).

In Arabidopsis, VIK is one of 66 proteins that are candidate interactors with the activated (phosphorylated) cytoplasmic domain of VH1/BRL2 (Ceserani et al., 2009). The other VH1/BRL2 interactors had annotated functions in protein degradation, vesicle trafficking, and signal transduction. Intriguingly, BRL2 is the only member of the BRI1-like subfamily of four LRR-RLKs that cannot directly bind BRs and cannot complement the loss of other family members, ruling out a direct role for VIK in BR perception (Clay and Nelson, 2002; Caño-Delgado et al., 2004). Nonetheless, Arabidopsis *vik* mutants display distorted responses to auxin and BRs (Ceserani et al., 2009). Thus, VIK has been speculated to play a role as an adaptor protein, acting as a scaffold linking the signal detected through VH1/BRL2 kinase to multiple targets (Ceserani et al., 2009). More recently, a tonoplast monosaccharide transporter from Arabidopsis (AtTMT1) also was shown to interact with VIK, implicating a further role in a mechanism regulating plant vacuolar Glc uptake (Wingenter et al., 2011). However, the PM-localized interaction between Pi17316 and StVIK suggests that it is less likely that *P. infestans* is trying to manipulate tonoplast monosaccharide transport through Pi17316. Therefore, we investigated what role VIK could play during the colonization of host plants by *P. infestans*.

VIK Is an S Factor

MAP3Ks, such as StVIK, may be involved in a diverse variety of signal transduction cascades from the plant PM to influence transcription in both growth and/or defense pathways. In order to determine why Pi17316 may be targeting VIK, two DNA fragments were selected (NbVIK I and II), using alignments of Arabidopsis, *N. benthamiana*, and potato VIK sequences, and cloned into a tobacco rattle virus (TRV)-based VIGS vector for transient silencing (Liu et al., 2002; Supplemental Figs. S8 and S9). Knockdown of the *NbVIK* transcript in VIGS plants was shown to be around 80% compared with the transcript abundance in TRV:GFP control plants (Supplemental Fig. S9B). Although efficient silencing levels were achieved, there was no detectable developmental phenotype observed in the *NbVIK* VIGS plants (Supplemental Fig. S9C). We showed that there was also no perturbation of the R3a- and Cf4-based cell death following the silencing of *NbVIK*, which is consistent with earlier observations

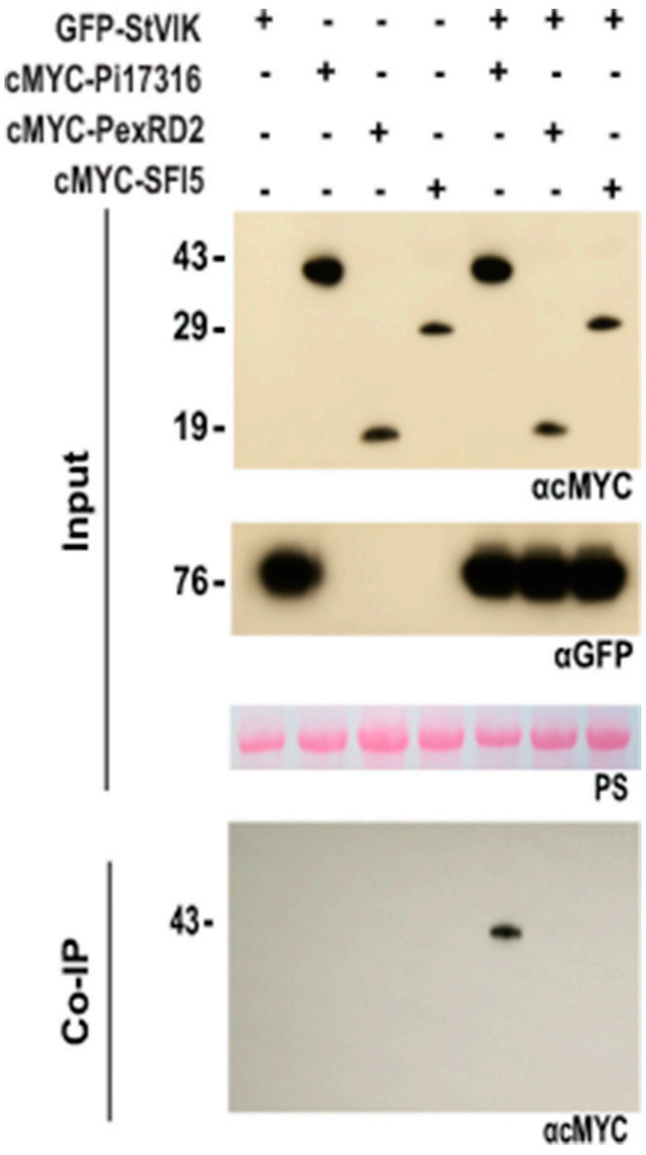

Figure 4. StVIK interacts with Pi17316 in planta. Coimmunoprecipitation (Co-IP) of protein extracts from agroinfiltrated leaves using GFP-Trap confirmed that GFP-tagged StVIK associated specifically with cMyc-Pi17316 and not with the cMyc-PexRD2 and cMyc-SFI5 controls. The expression of constructs in the leaves is indicated by +. Protein size markers are indicated in kD, and protein loading is indicated by Ponceau stain (PS).

that Pi17316 had no influence on these immune pathways (Supplemental Fig. S9D). Unexpectedly, *NbVIK* VIGS also had no measurable effect on ICD compared with the GFP control plants. Interestingly, however, measurements of both *P. infestans* lesion size and sporangia development on TRV:GFP- and TRV:NbVIK I- and II-expressing *N. benthamiana* revealed that silencing of *NbVIK* significantly reduced *P. infestans* colonization (Fig. 6). This indicates that VIK is required for infection by *P. infestans* and that it may be regarded as a host S factor. S factors can play multiple roles for the pathogen, such as in early pathogen establishment, pathogen sustenance, or suppression of immunity (van Schie and Takken, 2014). Since Pi17316 expression in planta suppressed ICD and boosted *P. infestans*, we next examined a possible role of VIK in plant immunity through transient overexpression.

Overexpression of VIK Perturbs ICD and Boosts *P. infestans* Colonization

To determine whether VIK's role as an S factor involves suppressing host immunity, GFP-StVIK was transiently coexpressed with INF1, R3a/AVR3a, and Cf4/AVR4 to examine the effect on these cell death pathways. Our data show that, like Pi17316, StVIK overexpression attenuated ICD but had no effect on R3a- and Cf4-based cell death (Fig. 7A). Both expression of the effector and the host target gave similar immunity phenotypes, implying that Pi17316 is unlikely to inhibit StVIK activity. We can conclude that StVIK, like Pi17316, behaves as a negative regulator of specific pathways in plant immunity. Therefore, the VIGS of *NbVIK* would accelerate ICD. However, it is possible that the silencing was insufficient to reveal enhanced or accelerated ICD. Overexpression of GFP-StVIK enhanced the ability of *P. infestans* isolate 88069 to colonize *N. benthamiana* compared with an EV control (Fig. 7B). This evidence strengthens our conclusion that StVIK acts as an S factor that negatively regulates immunity, although its exact mode of action remains elusive. As StVIK is the sole target of Pi17316, we next examined Pi17316 effector activity in the absence of VIK.

Pi17316 Requires VIK to Enhance Pathogen Colonization and Suppress ICD

As the transient expression of either Pi17316 or StVIK provided a benefit for *P. infestans* growth on host plants, we examined whether Pi17316 requires StVIK for this function. We performed infection assays following the transient expression of GFP-EV or GFP-Pi17316 in the TRV:*GFP* and TRV:*NbVIK* I and II VIGS backgrounds and examined the effect on *P. infestans* 88069's ability to colonize *N. benthamiana* plants. We observed that the control effector PexRD2 was able to significantly enhance *P. infestans* colonization regardless of *NbVIK* silencing. Crucially, we saw that Pi17316 was no longer able to enhance *P. infestans* colonization in the *NbVIK* VIGS background, whereas it did in the TRV:*GFP* control, indicating that Pi17316 requires the presence of StVIK for its role in virulence (Fig. 8A). As transient expression of Pi17316 or StVIK suppresses ICD, we next examined if Pi17316 could perturb ICD in the absence of VIK. We found that Pi17316 was no longer able to suppress ICD when *NbVIK* transcript levels were reduced significantly. However, the ability of AVR3a to suppress ICD in the *NbVIK* background was

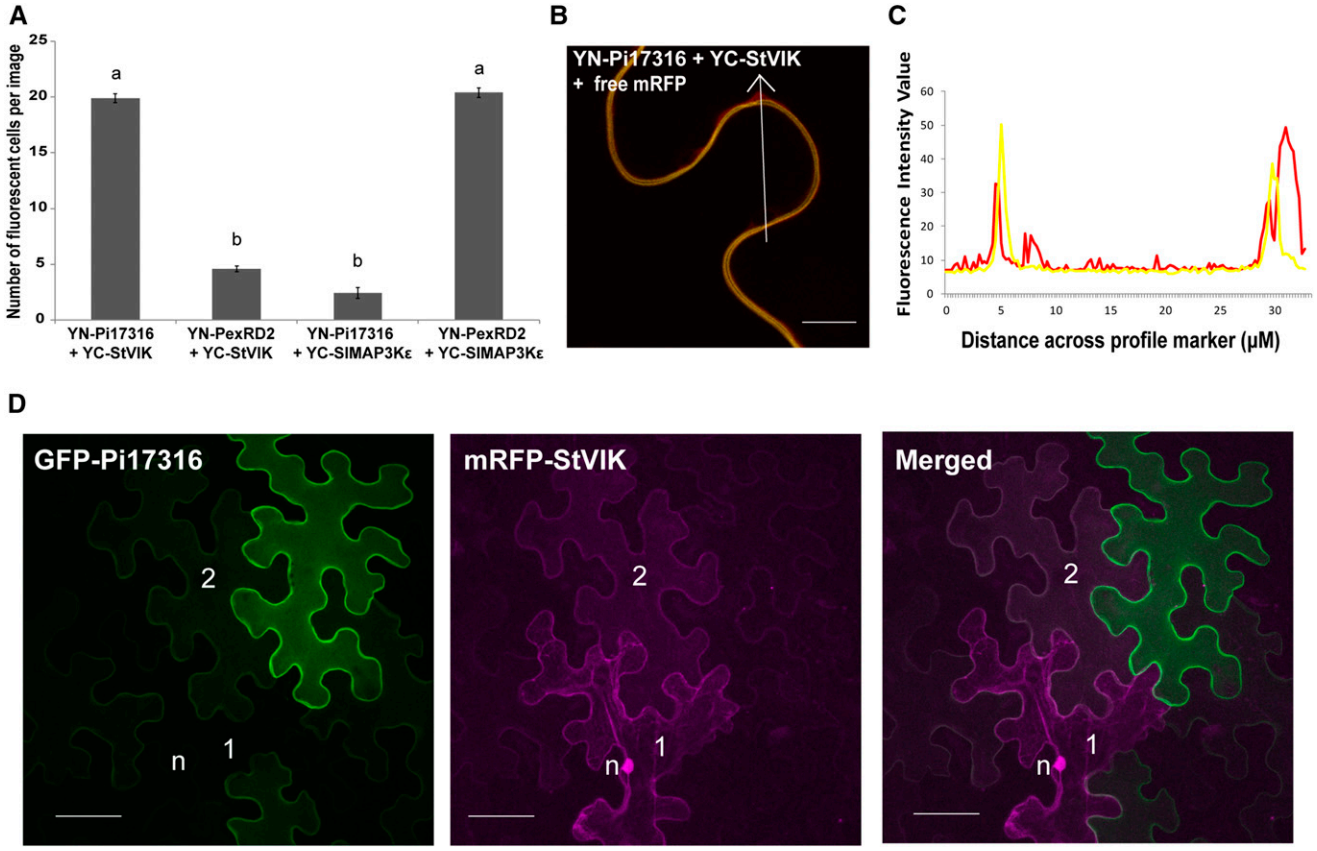

Figure 5. Pi17316 interacts with StVIK at the plant PM. A, Average number of fluorescent cells per image with YN-Pi17316 + YC-StVIK and YN-PexRD2 + YC:SlMAP3Kε, giving significantly more ($P \leq 0.050$, $n = 10$) reconstitution of YFP fluorescence than when noninteracting effector-interactor pairs (YN-PexRD2 + YC-StVIK and YN-Pi17316 + YC-SlMAP3Kε) were used. Lowercase letters denote statistically significant groups by one-way ANOVA, with pairwise comparisons performed with the Holm-Sidak method. Error bars show SE. B, Single optical slice image across PMs of two adjacent cells in the location indicated by the white arrow coexpressing YN-17316 + YC-StVIK and the free mRFP cytoplasmic marker. Bar = 20 μm. C, The plot of the profile indicates that the majority of the YFP fluorescence (yellow line) does not colocalize with the cytoplasmic marker (red line). D, GFP-Pi17316 coexpressed with mRFP-StVIK were imaged sequentially and merged using Omero software (https://www.openmicroscopy.org). Images were collected at 3 dpi at 40× magnification. 1 represents a cell where StVIK is present with little GFP-Pi17316, 2 represents a cell where both mRFP-VIK and GFP-17316 are expressed to similar levels, and n highlights the nucleus in cell 1. Bars 20 μm.

unaffected, indicating that the silencing of *NbVIK* specifically prevents the function of Pi17316 (Fig. 8B). This also may suggest that AVR3a acts downstream of Pi17316 to suppress ICD, perhaps consistent with its broader role in suppressing other immune responses, including Cf4-mediated cell death. AVR3a likely acts below a convergence point in these cell death signaling pathways.

The finding that Pi17316 interacts with a MAP3K in order to utilize or promote its ability to negatively regulate immunity shares similarities with the virulence function of the PM-localized *P. syringae* effector AvrB (Cui et al., 2010). Until publication of that study, plant pathogenic bacterial effectors were widely reported to block immune responses by inhibiting or degrading host target proteins required for plant immunity. Similarly, in oomycetes, various effectors suppress positive regulators of immunity, such as PexRD2 (King et al., 2014). However, AvrB targets and activates MPK4 to enhance plant susceptibility to the benefit of the bacterium (Cui et al., 2010). Similarly, Pi17316 targets StVIK, a negative regulator of plant defenses, presumably to activate it or to direct its activity to certain substrates. Future work will reveal the precise nature and consequences of Pi17316 interaction with StVIK upon the latter's kinase activity and substrate specificity.

To investigate the role of StVIK as an endogenous negative regulator of immunity, future work will focus on identifying its substrates. Existing literature implicates the BRI1-like receptor VH1/BRL2 as an interactor of VIK (Ceserani et al., 2009). Although silencing *NbVIK* did not measurably alter growth and development, it would be interesting to examine its role in regulating BR responses. It is possible that, through this route, Pi17316 could take advantage of antagonistic cross talk

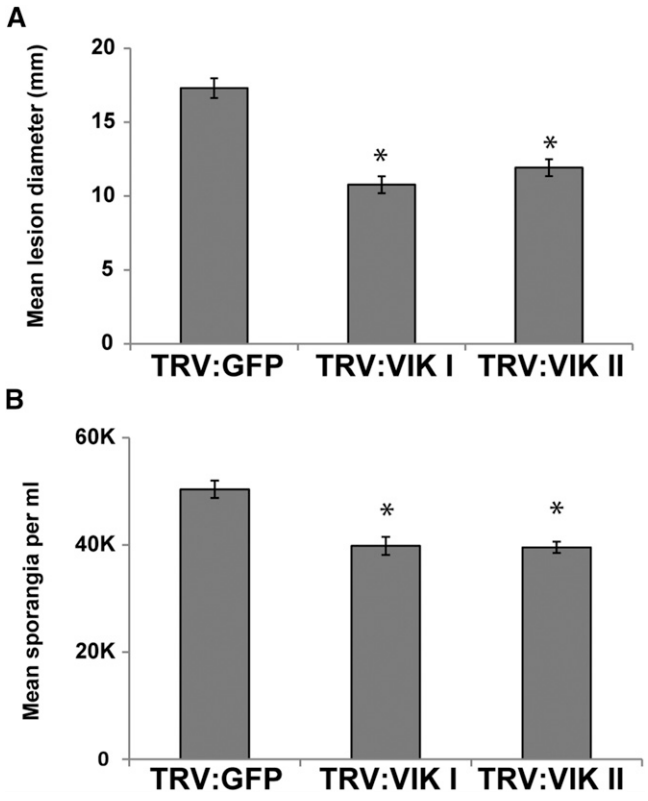

Figure 6. VIK is an S factor. A, Mean *P. infestans* lesion diameter (mm) on TRV:GFP, TRV:VIK I, and TRV:VIK II VIGS plants at 7 dpi. Error bars represent SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that TRV:GFP was significantly different from the lesion diameters measured for TRV:VIK I and TRV:VIK II VIGS (*, $P \leq 0.001$). B, Mean *P. infestans* spore counts per mL, washed from pools of TRV-infected leaves of each construct at 7 dpi. Error bars represent SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that the sporangia count from TRV:GFP control plants was significantly different from the sporangia measured from TRV:NbVIK I and TRV:NbVIK II VIGS plants (*, $P \leq 0.050$).

between the BR pathway and immunity, similar to the *P. infestans* effector AVR2 (Turnbull et al., 2017). However, as a Raf-like C1 MAP3K family member, interactions between StVIK and membrane-associated integrins also should be investigated (MAPK Group, 2002). Arabidopsis NDR1 is an integrin-like protein with well-characterized roles in immunity (Knepper et al., 2011), demonstrating that integrins may be valid targets to manipulate the regulation of disease resistance. As Pi17316 exclusively suppresses ICD, interaction with an integrin specifically associated with this immune pathway is possible. However, potential interactions between StVIK and the elicitin receptor ELR (Du et al., 2015), or signal transduction components associated with ELR activity, also should be investigated. Recently, a C1 MAP3K family member was shown to positively regulate immunity, promoting flg22-elicited PTI (Brauer et al., 2016). Conversely, StVIK reveals that C1 family members also can act as negative regulators of immunity.

A previous functional screen identified eight *P. infestans* SFI RXLR effectors that could significantly suppress flg22-dependent PTI reporter activation in tomato protoplasts (Zheng et al., 2014). This demonstrates that there is likely significant functional redundancy in the *P. infestans* effector repertoire. In support of this, through independent investigations, at least five *P. infestans* RXLR effectors have been identified, AVR3a, AVR2, Pi02860, SFI7/Avr3b, and now Pi17316, which suppress ICD. AVR3a has been shown to be essential for full pathogenicity (Bos et al., 2010). Future

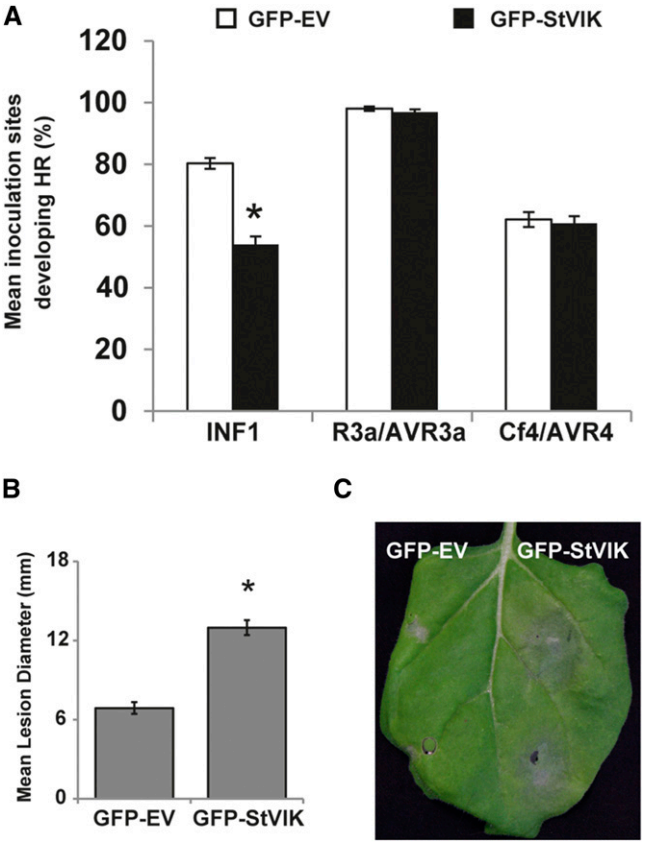

Figure 7. StVIK overexpression perturbs ICD and boosts *P. infestans* colonization of *N. benthamiana*. A, Average inoculations developing hypersensitive response (HR) lesions at 4 dpi for INF1, R3a/AVR3a, and Cf4/AVR4 (n = 128, 88, and 115, respectively) in seven biological repetitions. Error bars represent SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that ICD + StVIK was significantly different from ICD + EV (*, $P \leq 0.001$). B, Average *P. infestans* lesion diameter (mm) on sites of transient expression of either GFP-EV or GFP-StVIK measured at 7 dpi. The total number of samples per construct was measured (n = 160) in seven biological replicates. Error bars represent SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that *P. infestans* lesion diameter + StVIK was significantly different from lesion diameter + EV (*, $P \leq 0.001$). C, Representative image of *P. infestans* lesions at 7 dpi on sites of transient expression of either GFP-EV or GFP-StVIK.

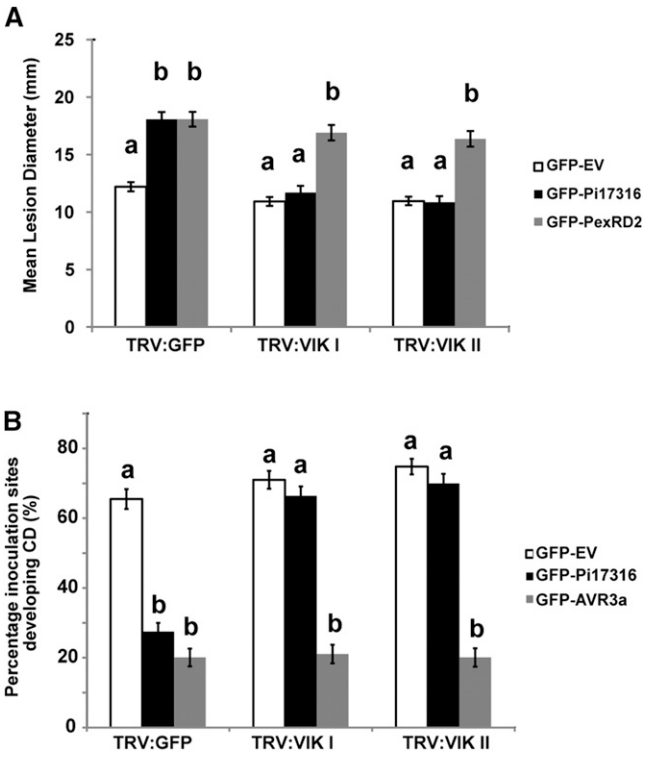

Figure 8. Silencing of *NbVIK* attenuates the ability of Pi17316 to suppress ICD and enhance *P. infestans* colonization in plants. A, Mean *P. infestans* lesion diameter measured at 7 dpi containing a sample number of roughly 150 for each construct in each VIGS background across four biological repetitions. Error bars represent SE. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method revealed that lesion diameter was significantly reduced when Pi17316 was expressed in the *NbVIK* VIGS backgrounds (a and b highlight two significance groups at $P \leq 0.001$). B, Percentage inoculations developing ICD in the presence of Pi17316 versus the EV in each VIGS background. The results represent a sample size of 121 across eight biological repetitions. One-way ANOVA using pairwise multiple comparison procedures with the Holm-Sidak method showed that ICD was significantly higher in the GFP-Pi17316-inoculated NbVIK VIGS backgrounds than in the TRV:GFP background (a and b highlight two significance groups at $P \leq 0.001$).

work will reveal whether Pi17316 and these other effectors are essential, given their redundant function in suppressing ICD. The known host protein interactors of these play distinct roles in regulating plant defense. AVR3a targets the ubiquitin E3 ligase CMPG1, which has been shown to be necessary for multiple cell death events, including ICD (Bos et al., 2010; Gilroy et al., 2011). AVR2 interacts with the Ser/Thr phosphatase BSL1, which is a positive regulator of growth and development controlled by the BR signaling cascade (Saunders et al., 2012). AVR2 perturbs ICD through up-regulation of the transcription factor *StCHL1* (Turnbull et al., 2017), potentially through its interaction with BSL1. NRL1 is a CULLIN3-associated ubiquitin E3 ligase targeted by effector Pi02860 that appears to be an S factor that can suppress ICD (Yang et al., 2016). The importance of elicitin perception in triggering defense to *P. infestans* has been demonstrated by transgenic overexpression of the corresponding receptor, ELR, leading to enhanced immunity (Du et al., 2015). It is perhaps unsurprising that multiple RXLR effectors with distinct host targets are employed to act redundantly to suppress ICD. Here, we have shown that Pi17316 targets StVIK, a MAP3K that also is an S factor and a negative regulator of ICD.

MATERIALS AND METHODS

Vector Construction

The *Phytophthora infestans* putative RXLR effector gene *Pi17316* was amplified with attB sites from cDNA generated from isolate 88069 to recombine into pDONR201 (Invitrogen) to generate entry clones. To make the stable overexpression vector PRI101-Pi17316, the effector was amplified with primers containing *Bam*HI and *Nde*I restriction sites and ligated into PRI101 using standard molecular biology techniques. The effector entry clones were recombined with pDEST32 (for Y2H; Invitrogen) and pB7WGF2 (for N-terminal EGFP fusion; Karimi et al., 2002). The effector entry clones also were recombined with pCL112 (for N-terminal YN fusion) or pCL113 (for N-terminal YC fusion) for BiFC (Bos et al., 2010) and with pGWB18 (for N-terminal tagging with the cMyc epitope; Nakagawa et al., 2007).

The full-length potato (*Solanum tuberosum*) VH1-interacting kinase was retrieved from pDEST32 (bait) of the original Y2H screen. The initially amplified coding sequence was amplified from potato cDNA with flanking attB sites. It was then modified to include an in planta spliceable intron 5 to make vector construction more stable in bacteria, and these products were recombined into pDONR201 (Invitrogen) to generate entry clones using the Gateway (Invitrogen) primer sequences shown in Supplemental Table S1. The StVIK was then recombined into pDEST22 (Y2H prey), pB7WGF2 (for N-terminal eGFP fusion; Karimi et al., 2002), and pCL112 (for N-terminal YN fusion).

Potato Transformation

The *Agrobacterium tumefaciens*-containing overexpression vector PRI101-Pi17316 was transformed into the potato cv E3 by microtuber disc transformation as described by Yang et al. (2016). Positive lines were confirmed by PCR with the forward primer of the 35S promotor and the gene-specific reverse primer of Pi17316. The presence and expression level of the transgene were analyzed by semiquantitative PCR (primers are shown in Supplemental Table S1). Seven-week-old potato plants were used for *P. infestans* inoculations.

Plant Production and Maintenance

Potato overexpression lines were grown in glasshouses in 16-h days at 22°C. Supplemental light was provided when the ambient light dropped below 200 W m^{-1}, and shading was provided when it was above 450 W m^{-1}. For transient overexpression assays, *Nicotiana benthamiana* plants were grown in general purpose compost under long-day glasshouse conditions of 16 h of light at 22°C, light intensity of 130 to 150 mE m^{-2} s^{-1}, and 40% humidity unless stated otherwise. *N. benthamiana* was used for *A. tumefaciens* infiltration/*P. infestans* colonization at 4 to 5 weeks old or at the four-leaf stage for VIGS experiments.

Agroinfiltration and Infection Assays

A. tumefaciens strain AGL1 transformed with vector constructs was grown overnight in YEB medium containing selective antibiotics at 28°C, pelleted, resuspended in infiltration buffer (10 mM MES and 10 mM MgCl acetosyringone), and adjusted to the required OD_{600} before infiltration into *N. benthamiana* leaves (generally 0.005 to 0.01 for imaging purposes, 0.002 for BiFC, 0.1 for infection assays, and 0.5 for hypersensitive response assays). For coexpression, *A. tumefaciens* cultures carrying the appropriate vector constructs were mixed prior to infiltration. *P. infestans* strain 88069 was used for plant infection and was cultured on rye (*Secale cereale*) agar at 19°C for 2 weeks. Plates were flooded with 5 mL of sterile water and scraped with a glass rod to release sporangia. The resulting solution was collected in a Falcon tube, sporangia

numbers were counted using a hemocytometer and adjusted to 50,000 mL^{-1}, and 10-mL droplets were inoculated onto the abaxial side of leaves of intact *N. benthamiana* plants stored on moist concertinaed tissue in sealed boxes. The lesions were measured at 6 to 7 dpi. For VIGS plants, the average lesion diameter was measured and compared with that of the GFP control plants at 7 dpi. *A. tumefaciens* transient assays in combination with *P. infestans* infection were carried out as described (McLellan et al., 2013). Cell death assays were performed and recorded as described previously using one-way ANOVA to assess statistical significance (Gilroy et al., 2011).

Confocal Imaging

N. benthamiana cells were imaged at 2 dpi using Leica TCS SP2 AOBS, Zeiss 710, and Nikon A1R confocal microscopes with Leica HCX PL APO lbd.BL 3/1.20 W and L 403/0.8, Zeiss PL APO 403/1.0, or Nikon 603/water-dipping objectives. GFP was excited by the 488-nm line of an argon laser, and emissions were detected between 500 and 530 nm. mRFP was excited with 561 nm, and its emissions were detected between 600 and 630 nm. The pinhole was set to 1 airy unit for the longest wavelength fluorophore. Single optical section images and z-stacks were collected from leaf cells expressing low levels of the protein fusions to minimize the potential artifacts of ectopic protein expression. Images were projected and processed using the Leica LCS, Zen 2010, and NIS-Elements software packages. Subsequent image processing for figure generation was conducted with Adobe Photoshop CS2 and Adobe Illustrator. The m-Turquoise-Lti6b, histone 2B fused to mRFP, and free RFP markers used were described previously (Kurup et al., 2005; Goedhart et al., 2010; Wang et al., 2017).

Y2H and Coimmunoprecipitation

A Y2H screen with pDEST32-Pi17316 was performed in *Saccharomyces cerevisiae* strain MsV203 as described (McLellan et al., 2013) using the Invitrogen ProQuest system. The full-length coding sequence of the candidate interacting prey sequence, StVIK (accession no. PGSC0003DMC400049296/PGSC0003DMT400072865), was cloned and retested with pDEST22-Pi17316; pDEST22-PexRD2 and pDEST22:SFI5 were used as controls to rule out the possibility that the observed reporter gene activation had resulted from interactions between the prey and the DNA-binding domain of the bait construct or the DNA-binding activity of the prey itself. *A. tumefaciens* strain AGL1 containing the fusion protein constructs was grown overnight in YEB medium containing selective antibiotics at 28°C, pelleted, resuspended in infiltration buffer (10 mM MES, 10 mM MgCl, and 200 mM acetosyringone), and adjusted to an OD of 0.5 before infiltration into *N. benthamiana* leaves. Forty-eight hours post infiltration, samples were taken and proteins were extracted. GFP-tagged StVIK fusions were immunoprecipitated using GFP-Trap-M magnetic beads (Chromotek). The resulting samples were separated by PAGE and western blotted. Immunoprecipitated GFP fusions and coimmunoprecipitated c-Myc fusions were detected using appropriate antisera (Santa Cruz Biotechnology).

VIGS

VIGS constructs were made by cloning two 250-bp PCR fragments of *NbVIK* (accession no. Niben101Scf00850g01028) from *N. benthamiana* cDNA and cloning into pBinary TRV vectors (Liu et al., 2002) between *Hpa*I and *Eco*RI sites in the antisense orientation. BLAST analysis of this sequence against the *P. infestans* genome (https://www.ncbi.nlm.nih.gov/bioproject/17665) did not reveal any matches that could initiate silencing in the pathogen. A *TRV* construct expressing GFP described previously was used as a control (McLellan et al., 2013). Primer sequences are shown in Supplemental Table S1. The two largest leaves of four-leaf-stage *N. benthamiana* plants were pressure infiltrated with LBA4404 *A. tumefaciens* strains containing a mixture of RNA1 (OD = 0.4) and each NbVIK VIGS construct or the GFP control at OD = 0.5 each. Plants were used for assays or to check gene-silencing levels by reverse transcription quantitative PCR 2 to 3 weeks later.

Gene Expression Assay

RNA was extracted using a Qiagen RNeasy Kit with on-column DNA digestion steps according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 mg of RNA using SuperScript II RNase HReverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Detection and data acquisition were performed by reverse transcription quantitative PCR using Power SYBR Green (Applied Biosystems) and run on a Chromo4-real time detector with a PTC-200 thermal cycler (MJ Research) using Opticon Monitor 3.1.32 software (all Bio-Rad Laboratories). Reactions were incubated at 95°C for 15 min before 40 cycles of 95°C for 15 s and 60°C for 1 min and plate reading. A subsequent melting curve was performed on every run: 58°C and 95°C, with plate read every 1°C and hold for 5 s. Data were analyzed using the Delta Delta Ct method (McLellan et al., 2013) with expression normalized to a housekeeping gene (*Actin A* for *P. infestans* or *Elongation Factor 1a* for *N. benthamiana*). Primer pairs (Eurofins MWG operon) were designed outside the region of cDNA targeted for VIGS to avoid the detection of products encoded in the viral vector. All primers are shown in Supplemental Table S1. Primer design was based on sequence information from the Sol Genomics Network (Fernandez-Pozo et al., 2015) at www.solgenomics.net and facilitated by the use of Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012; http://primer3.ut.ee/) and NetPrimer software (Premier Biosoft).

Accession Numbers

Accession numbers are as follows: AtVIK, At1g1400; StVIK, PGSC0003DMC400049296/PGSC0003DMT400072865; and NbVIK, Niben101Scf00850g01028.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of *Pi17316* in a *P. infestans* infection time course on potato.

Supplemental Figure S2. Protein stability of GFP-Pi17316 in *N. benthamiana*.

Supplemental Figure S3. Stable expression of Pi17316 in potato and susceptibility to *P. infestans*.

Supplemental Figure S4. Cell death responses were not influenced by transient expression of Pi17316.

Supplemental Figure S5. The flg22-induced PAMP response was not perturbed in transgenic Pi17316 potato cv E3.

Supplemental Figure S6. Sequence alignment of Arabidopsis, potato, and *N. benthamiana* VIK1 proteins.

Supplemental Figure S7. Representative GFP-StVIK, BiFC images and profiles, and construct stability.

Supplemental Figure S8. Nucleotide alignment of Arabidopsis, potato, and *N. benthamiana* VIK sequences.

Supplemental Figure S9. NbVIK constructs, silencing efficiency phenotypes, and cell death responses.

Supplemental Table S1. Primers used in this study.

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